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A retrospective serological survey on human babesiosis in Belgium

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Abstract

In Europe, most clinical babesiosis cases in humans have been attributed to *Babesia divergens* and *Babesia* sp. EU1. *Babesia microti* infection of humans occurs mainly in the United States; although a case of autochthonous *B. microti* infection and serological evidence of infection have been reported in Europe. The Indirect Fluorescent Antibody Test was used to screen sera from 199 anonymous Belgian patients with history of tick bite and clinical symptoms compatible with a tick-borne disease. The serological screen detected positive reactivity in 9% (n = 18), 33.2% (n = 66), and 39.7% (n = 79) of the samples against *B. microti*, *B. divergens*, and *Babesia* sp. EU1, respectively. Thus, evidence of contact among three potentially zoonotic species of *Babesia* and humans has been confirmed in Belgium. Preventive action and development of better diagnostic tools should help in prevention of clinical cases and to clarify the true burden of such infection for individuals and public health.

Keywords: *Babesia* spp., Belgium, human, serology, zoonosis
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Introduction

Babesiosis is a tick-borne disease caused by different species of intraerythrocytic protozoa classified within the genus *Babesia*. In humans, clinical symptoms that develop early, or during a mild *Babesia* sp. infection, can be confused with those of other causes of undifferentiated febrile illness, showing high fever, headaches, and myalgia. In acute infection, anaemia, icterus, and haemoglobinuria are conspicuous, and this condition can be fatal. In Europe, three species with zoonotic capabilities have been described [1]. Most clinical cases have been attributed to *Babesia divergens*, a zoonotic species commonly found in cattle, primarily in splenectomised or immunocompromised individuals [2], although some cases have recently been reported in young immunocompetent patients [3]. Recently, a new potentially zoonotic species of *Babesia* has been described. *Babesia* sp. EU1 (proposed species nomenclature: *B. venatorum*) was identified for the first time in two asplenic human patients [4], but has also been found to infect roe deer (*Capreolus capreolus*) [5,6]. *Babesia* sp. EU1 is unable to reproduce in gerbils (*Meriones unguiculatus*), providing evidence, together with evidence of genotypic divergence, for recognition as a distinct species from *B. divergens* [4].

Human cases caused by a third species, *Babesia microti*, have been recorded in both spleen-intact and asplenic patients; it usually presents as a relatively mild infection, except in

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immunocompromised or elderly individuals. *B. microti* is a natural parasite of microtine rodents and occurs mainly in the United States. However, a case of autochthonous *B. microti* infection has been confirmed in a German patient with an acute myeloid leukaemia [7], and serological evidence of human *B. microti* infections in a number of different European countries has been reported (Table 1) [8–23].

In Belgium, *B. divergens* is known to be present in cattle in the south of the country, but the disease is considered to be absent from other regions [24]. The risk of human infection by *Babesia* parasites in Belgium is generally considered to be low. However, a recent study identified the potentially zoonotic *Babesia* species EU1 and *B. microti* in the *Ixodes ricinus* tick [8]. Together with an older report of a human clinical case of babesiosis [21], this study provided the impetus for an evaluation of potential human contact with zoonotic *Babesia* parasites in Belgium. The study was conducted by performing a retrospective serological survey on samples obtained from humans in Belgium with a history of tick bite.

Materials and methods

Human serum samples used in this study were collected and sent by physicians to the laboratory for vector-borne diseases in Neder-over-Heembeek, Belgium. In total, 199 sera from anonymous Belgian patients with a history of tick bite in the month prior to the onset of clinical symptoms compatible with tick-borne disease (mainly fever and fatigue) were selected at random from samples obtained between 2005 and 2010 (with the approval of the ethical committee of the Liège University Hospital, reference B707201010146).

An indirect fluorescent antibody test (IFAT) was used to screen for the presence of antibodies against *B. divergens, Babesia* sp. EU1, and *B. microti* in the 199 human serum samples. A commercially available IFAT kit (Fuller Laboratories, Worcester, MA, USA) was employed to detect IgG against *B. microti*. According to the manufacturer's specifications, samples were scored as positive at a 1:64 or greater dilution. Positive and negative controls provided by the manufacturer and an additional positive control provided by the Reference Diagnostics Laboratory of the Center for Global Health (CDC, Atlanta, GA, USA), were used to confirm test accuracy.

The IFAT test for *B. divergens* was performed according to Chauvin *et al.* [25], using as the antigenic source, blood of a gerbil (*Meriones unguiculatus*) infected with *B. divergens* (Rouen87, clone F5) initially isolated from an acute human case of babesiosis. *Babesia* sp. EU1 antigen was derived from a cloned parasite line (C201A), initially isolated from roe deer and cultivated *in vitro* in sheep erythrocytes [5]. Serum samples from both species were screened at dilutions of 1:8 and 1:16, and were scored as positive at a 1:16 or greater dilution. Positive control sera were collected from *B. divergens*-infected cattle in France [26]. The human positive control serum was a kind gift from Dr. Maija Lappalainen, Helsinki University (HusLab; Finland), and originated from a fatal clinical case attributed to *B. divergens* in a 53-year-old man [27]. This serum was first screened for reactivity against *B. divergens* and *Babesia* sp. EU1 and demonstrated positive seroreactivity against *B. divergens* at a titre of 1:512. Human negative control sera were selected from a previous study (E. Moreau, unpublished data). Serum obtained from a sheep experimentally infected by *Babesia* sp. EU1 (clone C201A) was used as positive control for the *Babesia* sp. EU1 test (E. Moreau, unpublished data). Cross reactivity of

TABLE 1. Prevalence of zoonotic *Babesia* spp. (*B. divergens*, *Babesia* sp. EU1, and *B. microti*) in ticks, animals, and humans in Europe

Source	PCR		Serology (IFAT)	Country	Reference
Feeding ticks collected on:	Dog-Cat	1.1% (*B. microti*, *Babesia* sp. EU1)		Belgium	[8]
	Dog	< 1% (*B. divergens*, *Babesia* sp. EU1)		UK	[9]
	Deer	2.1% (*B. divergens*, *Babesia* sp. EU1)		Belgium	[10]
	Deer	4.8% (*B. microti*, *Babesia* sp. EU1)		Germany	[11]
	Bovine	14.6 % (*Babesia* sp. EU1)		Belgium	[12]
	Human	9.1% (*B. microti*)		The Netherlands	[13]
Questing ticks:		2.5% (*B. microti*, *Babesia* sp. EU1)		Germany	[11]
		0.8–11% (*B. microti*)		Switzerland	[14]
		1.25% (*Babesia* sp. EU1)		France	[15]
		1.1% (*B. microti*, *B. divergens*, *Babesia* sp. EU1)		The Netherlands	[16]
Bovine (*B. divergens*)			10.7–20%	Belgium	[12]
			27%	Norway	[17]
Deer (*Babesia* sp. EU1)		26.9%		Poland	[18]
			58.4%	France	[19]
		89.5%		Germany	[11]
Rodent (*B. microti*)		11.8-15.9%		Slovenia	[20]
Human			7.8% (*B. microti*)	Belgium	[21]
			5.4% (*B. microti*)	Germany	[22]
			1.5% (*B. microti*)	Switzerland	[14]
			6.2% (*B. divergens*)	Germany	[22]
			17.7% (*B. divergens*)	Slovenia	[23]

IFAT, indirect fluorescent antibody test; PCR, polymerase chain reaction.

sera among IFATs for *B. divergens, Babesia* sp. EU1, and *B. microti* was tested by reaction of positive serum controls against the respective antigen preparations. The blue-fluorescent 4, 6-diamindino-2-phenylindole nucleic acid stain (DAPI) was used to highlight the presence of parasite nuclei on examination of IFAT slides. Slides were mounted in 10 µL of mounting medium containing 50% glycerol in phosphate-buffered saline, DAPI at 1 µg/mL, and phenylenediamine at 1 mg/mL, prior to the addition of a coverslip.

A multivariate logistic regression was used to investigate possible effect of location, age, and sex of patients on their serological status for *Babesia* spp. infection (STATA/SE Acad. 12, StataCorp, LP, College Station, TX, USA).

Results

A panel of human sera from 199 patients with history of a recent tick bite was serologically screened. Ninety-five female and 103 male patients (1 unknown), mainly adults from 10 different Belgian provinces, were included in this study (Table 2).

Positive reactivity against *B. microti* was detected in 9% (n = 18) of the samples at a titre $\geq$1:64. Titration of *B. microti*-positive serum samples generated a range between 1:64 and 1:512. The percentage of samples displaying positive reactivity against *B. divergens* and *Babesia* sp. EU1 at a 1:16 dilution was 33.2% (n = 66) and 39.7% (n = 79), respectively. The percentage of sera displaying positive reactivity for *B. divergens* or *Babesia* sp. EU1 increased to 38.7% (n = 77) and 43.2% (n = 86), respectively, of the samples, if the cut-off for a positive reaction was taken at a 1:8 dilution. Co-infections were found for all three *Babesia* species (Table 3).

The reactivity pattern displayed by the human positive control sample and most of the *B. divergens* positive sera was observed as fluorescence concentrated at the apical poles on the divergent angle of dividing parasites (Fig. 1). DAPI staining showed that this structure did not co-localise with the nucleus of the parasite, and it was concluded that the serum antibody was reacting most strongly against antigen located to the apical complex (rhoptries/micronemes) (Fig. 2). This pattern of reactivity was distinct from that obtained with positive control serum derived from a bovine, where the pattern of reactivity was evenly distributed across the whole organism, with staining concentrated at the periphery in the absence of a detectable apical structure (Fig. 3). Reactivity against *Babesia* sp. EU1 antigen generated by positive sera from the samples of tick bite-associated patients showed the same punctate pattern of fluorescence as obtained with the *B. divergens* slides.

To test for species specificity of the IFAT, each positive control serum was cross-tested with each of the three antigen preparations representing the different species. The results showed that at the cut-off titre for positive reactivity (1:16 for *B. divergens* and *Babesia* sp. EU1, and 1:64 for *B. microti*), each control serum failed to react with antigen slides representing either of the other two species. This result indicates that if a serum sample from the obtained panel reacted with more than one species by IFAT, then it can be considered that the patient

TABLE 2. Sex, age and geographical locations associated with the 199 selected human patients

Sex		Age (years)		Location (provinces)	
Male	103	$\leq$ 20	12	Antwerp	68
Female	95	21–40	63	Flemish Brabant	31
Unknown	1	41–60	85	Walloon Brabant	18
Total	199	>60	37	Brussels	13
		Unknown	2	West Flanders	15
		Total	199	East Flanders	10
				Hainaut	13
				Liège	8
				Limburg	13
				Luxembourg	0
				Namur	4
				Unknown	6
				Total	199

TABLE 3. Results obtained for samples from 199 patients exposed to ticks from Belgium

	Seropositive samples *n* (%)
B. divergens	66 (33.2%)
Babesia sp. EU1	79 (39.7%)
B. microti	18 (9%)
Co-infections	
B. divergens / *Babesia* sp. EU1	38 (19.1%)
B. microti / *B. divergens*	4 (2%)
B. microti/ *Babesia* sp. EU1	4 (2%)
B. microti / *B. EU1* / *B. divergens*	2 (1%)
Babesia spp.	113 (56.8%)

Seropositivity was defined using the following cut-off titres: *B. microti* IgG IFAT $\geq$1:64; *B. divergens* IgG IFAT $\geq$1:16; *Babesia* sp. EU1 IgG IFAT $\geq$1:16.
IFAT, indirect fluorescent antibody test.

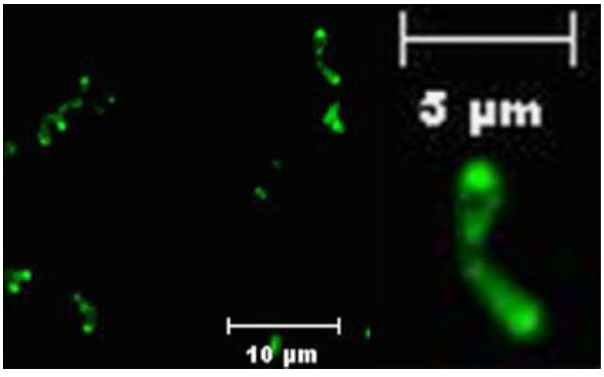

FIG. 1. Indirect fluorescent antibody test-reactivity of serum from a human clinical case of babesiosis in Finland [27] at dilution 1:8 using *B. divergens* parasites as antigen.

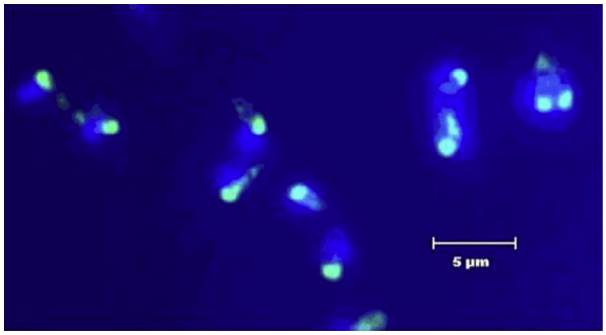

FIG. 2. 4, 6-diamindino-2-phenylindole nucleic acid stain (DAPI) staining of serum from a human clinical case of babesiosis in Finland [27] at dilution 1:8 using *B. divergens* parasites as antigen.

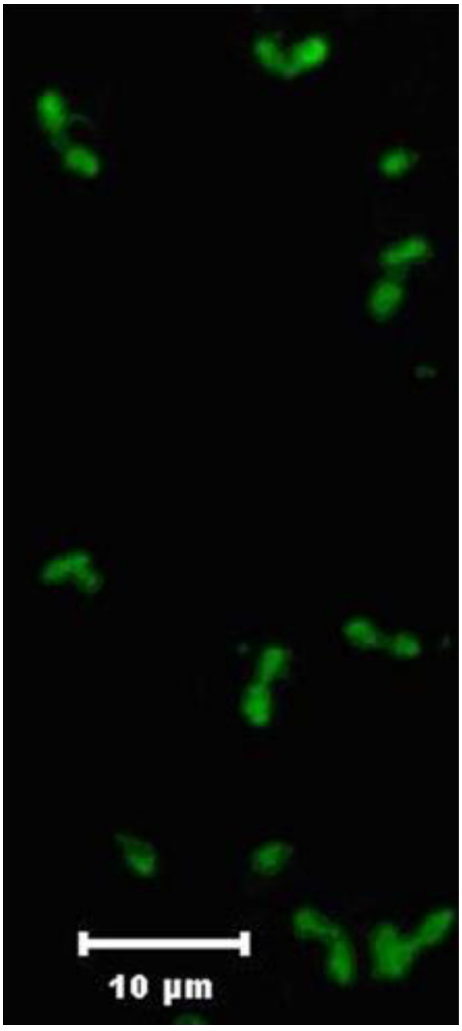

FIG. 3. Reactivity of serum (dilution 1: 80) obtained from cattle in France infected with *B. divergens* [26] using a *B. divergens* IFA test.

had been infected by two or more *Babesia* parasite species, at the same time or in succession.

Multivariate logistic regression showed an absence of a significant effect related to gender, whatever the *Babesia* spp (Table 4). However, it did indicate that the risk of infection for *Babesia* sp. EU1 per year may decrease as age increases; OR for the category of patients older than 60 years is 0.21 (95% CI 0.05–0.93); p 0.04. A higher risk of contact with *B. microti* was indicated for Namur province, as a significant association between positive reactivity and Namur province was identified using Antwerp province as reference; OR = 47.43 (95% CI 3.40–661.85); p 0.004.

Discussion

The retrospective serological survey performed in this study is unique, as it focused on three potential zoonotic *Babesia* species in Europe using a species-specific IFAT. The seroprevalence shown for *B. microti* (9%) is in agreement with other European surveys using samples from patients with a history of tick bite or suspected to be suffering from another tick-borne disease: 11.1% in Germany [22] and 7.8% in Belgium [21].

The seroprevalence of 33.2% for *B. divergens* is higher than previous surveys performed on samples from foresters in Slovenia (17.7%) [23] or patients with recorded tick bites in Germany (6.2%), while a recent survey in France generated a very similar level of seropositive samples (E. Moreau, unpublished data). Although an overestimation of the seroprevalence may be possible based on subjective scoring of equivocal IFAT reactivity as a positive, steps were taken to minimize this by performing two independent readings and discarding results obtained at a lower dilution than the defined cut-off titre. The source of antigen may also be a cause of variation among seroprevalence surveys. Although no difference was seen when antigen from human and bovine origin were compared, a higher end-point titre has been obtained using antigen of human origin [28]. Therefore, use of *B. divergens* of human origin as the source of parasite antigen may result in a higher seroprevalence percentage relative to bovine-derived antigen using the same titre cut-off point for positivity. Furthermore, seroprevalence for tick-borne infections will clearly be skewed toward positive reactivity when using samples derived from patients with a history of a tick bite and clinical symptoms compatible with tick-borne disease, compared to surveys incorporating samples from a wider range of human subjects. However, it has been suggested that, in Europe, human babesiosis may occur more often than previously indicated based on the results of the few serological surveys performed to date [14,22,28].

The IFAT reactivity pattern associated with human sera reacting against *B. divergens* parasites was found to be

TABLE 4. Risk or protective factors associated with *Babesia* spp. seropositivity in Belgium according to multivariate logistic regression

Babesia spp.	Variable		*n*	OR (95% CI)	p
Babesia sp. EU1	Gender	Female	38	Reference	
		Male	41	0.87 (0.45–1.65)	0.66
	Age of patients, years	≤20	7	Reference	
		21–40	27	0.57 (0.15–2.19)	0.42
		41–60	36	0.54 (0.14–1.97)	0.35
		>60	9	0.21 (0.05–0.93)	0.04
	Location	Antwerp	29	Reference	
		Flemish Brabant	11	0.74 (0.29–1.85)	0.52
		Walloon Brabant	10	1.72 (0.59–5.04)	0.32
		Brussels	5	0.76 (0.22–2.62)	0.22
		West Flanders	7	1.07 (0.33–3.48)	0.91
		East Flanders	2	0.38 (0.07–2.00)	0.26
		Hainaut	6	1.34 (0.38–4.70)	0.65
		Liège	2	0.20 (0.02–1.73)	0.14
		Limburg	5	0.90 (0.25–3.26)	0.87
		Namur	1	0.63 (0.05–6.83)	0.71
B. microti	Gender	Female	14	Reference	
		Male	4	3.35 (0.94–11.94)	0.06
	Age of patients, years	≤40[b]	3	Reference	
		41–60	11	3.24 (0.79–13.32)	0.10
		>60	4	1.82 (0.35–9.59)	0.48
	Location	Antwerp	4	Reference	
		Flemish Brabant	4	2.55 (0.55–11.88)	0.23
		Walloon Brabant	2	1.56 (0.25–9.75)	0.63
		Brussels	0	[a]	
		West Flanders	1	0.84 (0.08–8.54)	0.88
		East Flanders	2	3.34 (0.49–22.91)	0.22
		Hainaut	0	[a]	
		Liège	0	[a]	
		Limburg	1	1.06 (0.10–11.17)	0.96
		Namur	3	47.43 (3.40–662)	0.004
B. divergens	Gender	Female	30	Reference	
		Male	36	0.59 (0.30–1.16)	0.13
	Age of patients, years	≤20	3	Reference	
		21–40	15	0.87 (0.22–3.55)	0.85
		41–60	32	1.07 (0.28–4.05)	0.92
		>60	14	1.49 (0.35–6.37)	0.59
	Location	Antwerp	22	Reference	
		Flemish Brabant	6	0.59 (0.21–1.68)	0.32
		Walloon Brabant	6	1.09 (0.35–3.33)	0.89
		Brussels	5	1.24 (0.35–4.35)	0.74
		West Flanders	6	1.55 (0.47–5.14)	0.47
		East Flanders	3	0.95 (0.22–4.14)	0.94
		Hainaut	3	0.74 (0.18–3.08)	0.68
		Liège	4	2.43 (0.53–11.07)	0.25
		Limburg	4	0.89 (0.21–3.76)	0.88
		Namur	3	7.11 (0.66–76.50)	0.11

n, number of seropositive patients;.
[a]All patients from this province were seronegative.
[b]Categories of age ≤20 years and between 21 and 40 years were merged as all patients of the first category were seronegative.

predominantly located to the apical complex. This pattern may suggest the presence of immunodominant antigens localized in the apical complex of the parasite. *B. divergens* merozoite surface antigen (Bd37) is known to be a major immunodominant protein, and while this immunodominant antigen could be detected by immunoblotting with bovine serum samples, it was not detected with the human samples of this study (data not shown). The reason for this result is not clear, although polymorphism of Bd37 has been reported [29].

The reported study together with a related survey performed in France (E. Moreau, unpublished data) are the first to evaluate *Babesia* sp. EU1 seroprevalence using as antigen a confirmed *Babesia* sp. EU1 cloned line isolated from an *in vivo* infection. No clear evidence of cross reactivity was observed between antigen preparations employed for each IFAT when using positive control serum specific for each of the three species. Given the close phylogenetic relationship between *B. divergens* and *Babesia* sp. EU1, this result could be viewed as surprising. While Duh and collaborators [28] described an absence of cross reactivity of serum against *B. divergens* with *B. microti*, cross reactivity of sera between *B. divergens* and *Babesia* sp. EU1 has been reported [4,28]. It may be that, previously, IFAT positive results have scored potential *B. divergens* and *Babesia* sp. EU1 co-infection as cross recognition. In the present study, 41 patients positive for *Babesia* sp. EU1 were scored as negative for *B. divergens*. This result indicates that the antigenic epitopes predominantly recognized by these and the control sera have diverged between the two species to the extent that cross recognition does not occur. A confirmed lack of cross reactivity would lend further support to the description of *Babesia* sp. EU1 as a distinct species of *Babesia*.

The number of potential co-infections (n = 38) identified in this study based on co-recognition of both *B. divergens* and *Babesia* sp. EU1 by individual sera was high. Frequent co-infections with *B. divergens* and *Babesia* sp. EU1 may be possible, as these species share the same tick vector. In addition, the possibility of maintained transmission of *B. divergens* across tick stages and transovarially into the next generation could promote co-infection of ticks [10]. Furthermore, deer have been recognised as the main reservoir host for *Babesia* sp. EU1 and, while still controversial, may act as hosts for *B. divergens* [5]. The presence of the two *Babesia* species in the same reservoir host would increase the probability of co-transmission. Further studies are required to show that results based on recognition of both species by an antibody test correlate with molecular data validating co-infection of the mammalian host.

Multivariate logistic regression analysis showed that the risk of being seropositive for *B. microti* was higher in Namur province than the other provinces. This result has to be treated with caution, as only four samples from Namur were analysed. In addition, the apparent association indicating a lower chance of *Babesia* sp. EU1 infection in the older group of patients may be explained by differences in leisure activity or a greater awareness of tick-borne diseases by older patients, and consequently, faster removal of attached ticks that could reduce parasite transmission. Alternatively, differences in the acquired immune response may play a role, with adults more effective at reducing time of tick feeding, again reducing the chance of parasite transmission [30].

Unfortunately, clinical data and information about work and leisure activities of patients were not available, and data confidentiality did not allow determination of risk factors that may be associated with tick contact and transmission of infection.

At present, IFAT, although not ideal, is the best available test to evaluate the occurrence of *Babesia* spp. transmission to humans, since these parasites are usually eliminated rapidly by the immune system, leaving only a serological trace. Analysis using other serological tests, ELISA and Western blot, were attempted in this study, but the major immunodominant merozoite surface antigen could not be detected using the human sera (unpublished observation). To fully evaluate the level of exposure of the human population in Belgium to *Babesia* parasites, the development of a "home-made" IFAT test using molecularly validated European strain of *B. microti* and *Babesia* sp. EU1 derived from human cells as antigen is required. The paucity of European *B. microti* and *Babesia* sp. EU1 human clinical cases, however, means that routine provision of a standard antigen would be extremely difficult without an available culture system. Moreover, the use of the commercial IFAT kit based on a *B. microti* strain from the United States imposes significant costs and precludes the use of this test for a large-scale serological survey, which may lead to an underestimation of human *B. microti* infection in Europe. Such an underestimation could have potential consequences, given that it is now recognized that contamination of human blood with *B. microti* is a potential risk during transfusion and that *B. microti* infections are now notifiable in the United States [31].

In summary, the presence of the three potentially zoonotic species of *Babesia* in Belgium has been supported in this study, by provision of evidence for human contact with *B. divergens*, *Babesia* sp. EU1, and *B. microti*. Thus, in Belgium, as in other European countries, babesiosis can be considered as a potential threat for human health, especially in splenectomised and immunocompromised individuals. Preventive action against tick attachment together with improved awareness of babesiosis by physicians and development of better diagnostic tools should help in prevention of clinical cases and to assess the risk to public health.

Transparency Declaration

The authors declare that they have no conflicts of interest.

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